DEvelopment of a quick Monitoring index as a tool to assess Environmental impacts of TRAnsgenic crops (DEMETRA)

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Ente Parco Regionale Migliarino San Rossore Massaciuccoli

UNIVERSITÀ DEGLI STUDI FIRENZE
GESAAF
DIPARTIMENTO DI GESTIONE DEI SISTEMI AGRARI, ALIMENTARI E FORESTALI

DEvelopment of a quick Monitoring index as a tool to assess Environmental impacts of TRAnsgenic crops (DEMETRA)

Chapter 2

Pollen flow and breeding evaluation

2.2 - Breeding Evaluation

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Introduction

Gene flow between crops and wild species relatives has been occurring for thousands of years (Hancock et al. 1996, Ellstrand et al. 1999), but scientific attention on gene flow from crops to wild relatives and other crop populations is more recent, stimulated by concerns about the movement of transgenes (Snow and Morán-Palma 1997, Hall et al. 2000, Ellstrand 2002). Regardless of whether transgenes are involved, the consequences of gene flow from crops can be problematic. Crops genes may replace wild genes, reducing the genetic diversity of wild populations. Crop genes may also flow to other crop varieties or land races, contaminating the recipient seed pools. Whether this genetic contamination is called “genetic pollution” or “adventitious presence”, it can have undesired consequences, reducing seed quality (Friesen et al. 2003), threatening food safety (NRC 2004) and organic food production, or harming indigenous cultures [North American Free Trade Agreement– Commission for Environmental Cooperation (NAFTA–CEC) 2004]. If the resulting hybrids have lower fitness than their wild parents, the wild populations may shrink, threatening the survival of the wild population (Ellstrand and Elam 1993, Levin et al. 1996). Alternatively, if the resulting hybrids have higher fitness than their wild parents, they may become invasive (Tiedje et al. 1989), replacing the wild population and other species in agricultural and natural areas. Domesticated plants represent lineages that diverged from their progenitors no more than a few thousand generations ago. There is no reason to assume that reproductive isolation should be absolute (Ellstrand et al. 1999). Whether the evidence is reviewed on a regional basis or a crop-by-crop basis, it is clear that spontaneous hybridization and introgression of genes from domesticated plants to wild relatives is a common characteristic of domesticated plant taxa (Ellstrand et al. 1999). A major issue for plant genetic engineering is the extent to which transgenes will escape

from cultivation and cause negative impacts in wild ecosystems (Rogers et al. 1995, Wolfenbarger and Phifer 2000). Gene flow to wild relatives occurs for nearly all crops in places where they are grown (Ellstrand et al.1999). However, it is of particular concern for forest trees because they are virtually undomesticated (Bradshaw and Strauss 2001), they have the potential for spatially extensive gene flow (Hamrick et al. 1992, Slavov et al. 2002), and they can have large effects on ecosystem processes and biological diversity when they are the dominant life form (Wells et al. 1986). The ecological impacts of transgenic trees will primarily depend on the traits conferred by the transgene and the environment in which the trees are grown (Mullin and Bertrand 1998, James et al. 1998). Risk assessment therefore requires detailed consideration of the specific ecological consequences of individual transgenes in different settings. However, gene flow is a prerequisite for most ecological impacts outside of plantations, so baseline estimates of introgression will apply to most environmental risk assessments for transgenic trees (Ellstrand 2001, Muir 2001). The poplar has become a model tree species in genetic engineering as it can easily be transformed and clonally propagated and has a small genome size (Boerjan 2005). Tree growth, agronomic traits, and timber quality can be improved through genetic engineering (Pullman et al. 1998), thereby avoiding the long reproductive cycles of conventional breeding (Mathews and Campbell 2000). The potential environmental hazards linked to GM trees differ from those associated with transgenic crop plants at both spatial and temporal scales (van Frankenhuyzen and Beardmore 2004) because trees are long-lived perennials, unlike annual crop plants.

The evaluation of breeding was focused on two species: oilseed rape and poplar. The choice was conditioned by the presence of wild relatives with the same periods of phenological development for these two species in the study areas of the DEMETRA project.

Results and discussion

1. Breeding evaluation in poplar

In the protected area of Regional Park of Migliarino – San Rossore – Massaciuccoli two permanent study Areas 1 and 2, 8 Km faraway, are detected and placed in different ecosystems (Chapter 1).

Fig. 1. Study Area 1 (Massaciuccoli Lake).

The Study Area 1 is a wetland habitat, where single trees and small groups of poplar are scattered along the shores of the Massaciuccoli Lake (Fig. 1). In relation to the herbaceous stratum, the observations made so far show some variability in the distribution, probably due to the micro topological characteristics of the site. Sedges and reeds communities growing in the area have to be referred to the class *Phragmito-Magnocaricetea* Klika. This site is characterized by many rare or

threatened species, such as: *Periploca graeca* L., *Leucojum aestivum* L., *Hibiscus palustris* L. (Chapter 3). A total of 32 poplars were identified and their position was collected by GPS. Plant tissue was taken from each poplar for subsequent laboratory analysis.

The Study Area 2 is a naturally-originated mixed forest stand, in proximity of Serchio river, where the prevailing tree species are poplar, *Fraxinus angustifolia* and *Alnus glutinosa*, the latter in the lower strata (Chapter 1). According to the vegetation analysis, the woods with poplar can be referred to *Carici remotae-Fraxinetum oxycarpae* Pedrotti (Chapter 3). In this area within an experimental plot, 7,000 m^2 large, all poplar trees (30 individuals) were identified and their position was collected by GPS (Fig. 2). Even in this case, plant tissue was taken from each poplar for subsequent laboratory analysis. The poplars in the Study Area 2 are morphologically classified as belonging to the *Populus alba* (white poplar) species and hybrid *P. x canescens* (gray poplar). *P. alba* and *P. tremula*, parental of the hybrid *P. x canescens*, are two ecologically divergent species that hybridize frequently in Europe. In addition, the species of poplars sampled was identified by sequence analysis of the *trn*L-*trn*F cpDNA (chloroplast DNA) region (Table 1.).

Fig. 2. Study Area 2 (Serchio river).

Table 1. *Variable sites into trnL-trnF cpDNA region of considered poplar species.*

	Variable Sites[a]		
	198	279	478
P. alba	T	A	A
P. x canescens	T	A	A
P. tremula	T	A	G
P. nigra	T	A	A
P. deltoides	G	G	A
P. x euramericana	G	G	A

[a] Primers used as reported in Taberlet et al. 1998; amplification conditions and alignment sequences used as reported in Paffetti et al. 2007.

Fig. 3. Study Area 2 (Serchio river). In yellow color are reported the position of gray poplar.

Moreover, the poplars were genotyped using 10 nuclear microsatellites (nSSR) loci[1]. The nuclear microsatellites present unique allelic variant for each species. The results show that in the study plot 6 trees belong to gray poplar, including 5 females and 1 male, and 23 males and 1 female are white poplars (Fig. 3 and Table 2).

The cpDNA[2] haplotype variant is identical for all individuals, as the mother of the hybrid *P. x canescens* is *Populus alba*. The values of genetic diversity, given

here only as an average of the data obtained by 10 microsatellite loci, appear to be low (Table 3), but consistent with the data previously reported in literature for natural populations of the two species (Lexer et al. 2005).

Table 2. Number (ID), sex and molecular identification by cpDNA haplotype and nSSR genotyping of each poplar tree considered in the Study Area 2.

ID poplar tree	*Sex*	*cpDNA haplotype*	*nSSR genotyping*
P1, P2, P3, P4, P6	♀	TAA	*P. x canescens*
P10	♂	TAA	*P. x canescens*
P7, P8, P9, P11, P12, P13, P14, P15, P16, P17, P18, P19, P20, P21, P22, P23, P24, P25, P26, P27, P28, P29, P30	♂	TAA	*P. alba*
P5	♀	TAA	*P. alba*

Table 3. Genetic variability estimates. Number of alleles (N), number of rare alleles (N_{rare}), expected heterozygosity (H_e) and fixation index (F_{IS}).

	P. x canescens				*P. alba*			
	N	H_e	N_{rare}	F_{IS}	N	H_e	N_{rare}	F_{IS}
Mean	1.4	0.174	0.00	0.002	3.0	0.350	0.25	-0.001

We inferred spatial population structure using a Bayesian Monte Carlo Markov Chains method implemented in the Geneland package (Guillot et al. 2005) under the R Language. The results show that the population is divided into four clusters (Fig. 4). The clusters are genetically isolated, as indicated by the maps of posterior probability, but each cluster does not seem to be composed of trees related to each other. The cluster

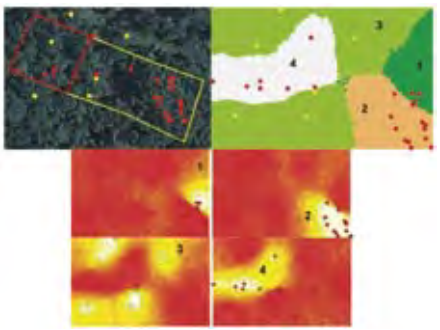

Fig. 4. Spatial organization into four clusters and maps of posterior probability of each cluster.

Fig. 5. Study Area 1 (Massaciuccoli Lake). Yellow dots represent the positions of gray poplars, red dots of white poplars and green dots of black poplars.

[1] Primers and amplification conditions used as reported in Tuskan et al. 2004 and Smulders et al. 2001.
[2] In poplar, the chloroplast in the chloroplast is inherited from the mother.

indicated by the number 3, is exclusively composed of *P. x canescens* trees. This cluster appears genetically more isolated from others.
In addition to the two poplar species, *P. alba* and *P. x canescens*, present in the Study Area 2, in the Study Area 1 trees attributable to the *P. nigra* (black poplar) morphology were identified. This was confirmed by the analysis of sequence data and genotyping (Fig. 5 and Table 4). As expected, the cpDNA haplotype of *P. nigra* is identical to *P. alba* haplotype, and black poplar presents the specific microsatellite allelic variants for the species. The individuals for this species show the higher levels of genetic diversity among all three species (Table 5).

Table 4. Number (ID), sex and molecular identification by cpDNA haplotype and nSSR genotyping of each poplar tree considered in the Study Area 1.

ID poplar tree	*Sex*	*cpDNA haplotype*	*nSSR genotyping*
PM1, PM2, PM3, PM5, PM6, PM10, PM17, PM20, PM29, PM30, PM31	♂	TAA	*P. alba*
PM12, PM23, PM25, PM27	♀	TAA	*P. alba*
PM4, PM11, PM13, PM16, PM18, PM19, PM21, PM24, PM28	♂	TAA	*P. x canescens*
PM22, PM26, PM32	♀	TAA	*P. x canescens*
PM8, PM15	♀	TAA	*P. nigra*
PM7, PM9, PM14	♀	TTA	*P. nigra*

Table 5. Genetic variability estimates. Number of alleles (N), number of rare alleles (N_{rare}), expected heterozygosity (H_e) and fixation index (F_{IS}).

	P. alba				*P. x canescens*				*P. nigra*			
	N	H_e	N_{rare}	F_{IS}	N	H_e	N_{rare}	F_{IS}	N	H_e	N_{rare}	F_{IS}
Mean	3.6	0.445	0.22	0.012	3.6	0.550	0.17	0.011	3.3	0.565	0.00	0.003

The spatial structure population has a division of population into three clusters. In this case, as evidenced by the maps of posterior probability and the values of F_{ST}, only the cluster 1, comprising the *P. nigra* tree species, is genetically isolated from the others. On the contrary, gene flow is evident between the other two clusters consisting of *P. alba* and *P. x canescens* trees. The different clusters are constituted by unrelated individuals between them (Fig. 6).
Both the Study Area are adjacent to poplar plantations. In particular, the Study Area 2 is adjacent to "Triplo" multiclonal plantation and to "Onda" multiclonal plantation. The "Triplo" is a hybrid of *Populus deltoides* (mother) and *Populus nigra* (father), defined *P. x euramericana*. While "Onda" is a *P. deltoides* selection. The Study Area 1 is adjacent to

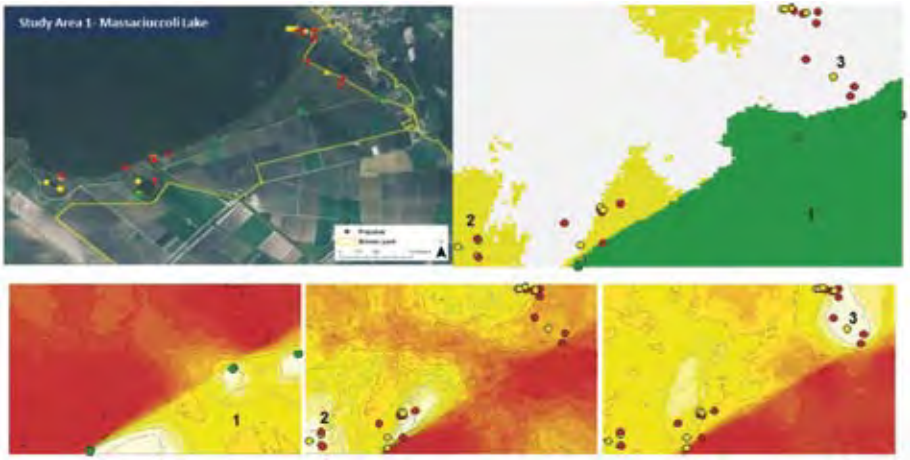

Fig. 6. Spatial organisation into three clusters and maps of posterior probability of each cluster.

poplar multiclonal plantation, a *P. deltoides* selection. The sequence analysis shows that the mother of cultivated varieties is *P. deltoides* and genotyping allows the identification of each cultivated variety (Table 6).

After analyzing the species phenology, in particular, after confirming the correspondence of pollen production period from plantations and the receptivity ovary period of female trees present in natural populations, the genotyping of the offspring, germinated seeds, was performed by the same nuclear microsatellites loci used for identification.

Table 6. Number (ID), sex and molecular identification by cpDNA haplotype and nSSR genotyping of each poplar plantation.

ID poplar tree	*Sex*	*cpDNA haplotype*	*nSSR genotyping*
"Onda" plantation (Study Area 2)	♀	GGA	*P. deltoides*
"Triplo" plantation (Study Area 2)	♀	GGA	*P. x euramericana*
Poplar plantation (Study Area 1)	♀	GGA	*P. deltoides*

We conducted paternity assignment using all ten analysed nSSR loci by standard maximum-likelihood methods implemented in CERVUS 3.0 (Marshall et al. 1998). Critical likelihood values (LOD-scores) yielding 95% confidence in assignments were obtained using simulations.

In Study Area 1, the crossings of PM 11 (gray poplar), PM 20 and PM 23 (white poplar) mother trees were identified (Fig. 7). The crossings involved male individuals belonging to both species, but, as expected, hybrids with black poplar were not detected. In Study Area 2, the crossings of P 1, P 2 and P 6 mother trees belonging to the species gray poplar with individuals of white poplar were identified. Only the P 2 mother tree produced offspring with the P 10 male tree of the same species. The P 5 mother tree, *P. alba,* produced offspring only with individuals of its own species (Fig. 8).

The P 3 mother tree, *P. x canescens*, produced offspring with individuals of *P. alba* species (Fig. 8). But unexpectedly the hybridization occurred between this tree and "Triplo"

Fig. 7. The crossings identified in the Study Area 1.

Fig. 8. The crossings identified in the Study Area 2.

plantation. The analysis of forest structural data (diameter and height trees) showed, that P 3 is a large tree which have not barriers to the pollen flow from the plantation due to competition with other crowns. Furthermore, thanks to the analysis of the wind direction and speed, it was possible to find that during the pollen diffusion and ovary receptivity the direction east-northeast to west-southwest of the wind favored pollen dispersion from F1.1 tree ("Triplo") to the P3 tree (Fig. 9).

This work confirms the presence of *P. x canescens* in naturally-originated stand. In addition, it was possible to identify the presence of the hybrid in the Massaciuccoli Lake. In the mixed forest the *P. x canescens* subpopulation appears spatially genetically isolated. While, there is not the same situation on the Lake. The *P. alba* and *P. x canescens* groups, in this case, present higher level of genetic variability and an evident gene flow. This is probably related to different environmental conditions. The detection of breeding, between the tree in Study Area 2 and the plantation, suggests the occurrence of a possible genetic exchange among a natural population and plantation which has to be carefully considered

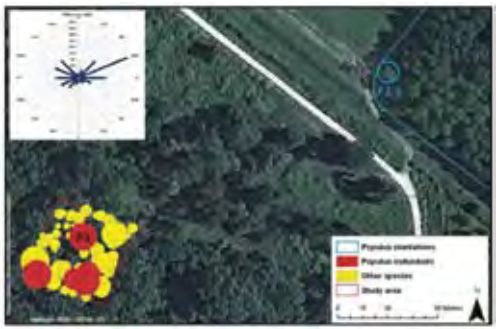

Fig. 9. Crossing between P 3 and F 1.1. poplar.

when poplar plantation are made close to natural environment in which wild relatives are present. The interspecific incompatibility in *Populus* has been extensively studied with the aim of obtaining hybrids for forest tree breeding programs. Hybrids are freely obtained between species within a section, but not between section. However, in the literature several examples reported the unilateral hybridization phenomena. For example Ronald (1982) reported that the cross of white poplar-aspen (*P. tremula*) hybrid female partner to *P. deltoides*, to *P. nigra* and to *P. x euramericana* produced a quantity of viable seeds and seedling.

2. Breeding evaluation in oilseed rape

Some wild plant species belonging to Brassicaceae have been selected in the protected areas of Regional Park of Migliarino – San Rossore – Massaciuccoli, to evaluate possible hybridization and gene flow. The choice of Brassicaceae has been done considering the pollinating fauna and the flowering period. *Sinapis arvensis* (Fig. 10) was consider as one possible candidate to hybridize with *Brassica napus* L. var. *oleifera* Del. (oilseed rape) (Chapter 3). Due to the close genomic relationship between these taxa, nSSR primers designed for different Brassicaceae species were tested to amplify both in *Brassica napus* and *Sinapis arvensis*. Therefore we looked for *S. arvensis* (field mustard) populations and oilseed *B. napus* cultivars which would hybridize in the field. To verify possible hybridization between *Brassica napus* and *Sinapis arvensis* 41 nuclear microsatellite markers were tested. These markers, derived from literature (Lowe et al. 2002, 2004, Szewc-Mc Fadden et al. 1996, Lagercrantz et al.1993) were designed on different species of Brassicaceae and were selected on the basis of their transferability. Only 10 primer pairs showed amplification in both *Brassica napus* and *Sinapis arvensis* samples. Among them, 2 primer pairs distinguished different alleles between *B. napus* and *S. arvensis*. In order to verify if hybridization occurs in the field, a sampling of field mustard (potential mothers) and oilseed rape (potential fathers) plants was performed (Fig. 10). Ten plants of field mustard were randomly chosen as mothers and georeferenced. All potential fathers of oilseed rape (442 individuals in total) were sampled within 3 m radius away from field mustard (mother) and their distance from mother as well as the

Fig. 10. Sinapis arvensis L.

Fig. 11. Experimental process using to breeding evaluation between oilseed rape and field mustard.

cardinal direction were registered (Figure 10). About 100 seeds per mother were collected by "Arasystem" (Betatech bvba) traps, soaked with a KNO_3 solution and posed in Petri dishes to germinate (Fig. 11).

The plants of wild mustard (indicated by the red color in Fig. 12) have allelic variants that distinguish them from two varieties of rapeseed cultivation (CV1 and CV2 indicated with black and yellow color in Fig. 12, respectively).

Furthermore, these cultivars can be distinguished by different allelic variants. It was thus possible to determine that most of the seeds appear to be the product of self-fertilization of *Sinapis arvensis*, as expected. But a smaller percentage of the seeds turns out to be the product of fertilization between different individuals of wild mustard, and between plants of wild mustard and rapeseed. In particular, the plant of wild mustard indicated with the number 3 and the plant indicated with the number 10 have produced hybrid offspring with rape plants of CV2.

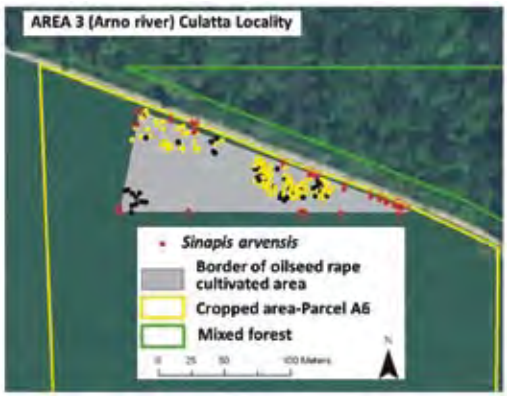

Fig. 12. Sinapis arvensis plants (mother) and oilseed rape (father): CV1 (black dots) and CV2 (yellow dots).

If GM oilseed rape is to be grown, the possibility of his modified trait being transferred to *S. arvensis* needs serious consideration as the species are widespread.

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